Separation of Fluorescent Antibody Conjugates into 7S and 19S Globulin Components by Gel Filtration.* (30290)

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Restrictions are imposed on the usefulness of immunofluorescent reagents by the occurrence of staining not attributable to combination of specific antibody with antigen. Mayersbach and Schubert(1) considered this nonspecific staining (NSS) as due to the affinity of positively charged protein molecules of the substrate for negatively charged molecules of the conjugate. The explanation was supported by their observation that NSS of tissue sections by conjugates was decreased by rinsing the sections in alkaline solution. The argument was strengthened by other studies(2,3) which indicated that the negative charge of serum protein molecules was increased by conjugation with fluorescein and that those molecules with the greatest negative charge produced the most NSS.

While it is known that all types of serum proteins do not couple with fluorescein at the same rate(4), no comparison has been made of the rates of labeling of the 7S and 19S globulins that are prominent in fractions of antisera commonly employed for conjugation. The present study was designed to learn if NSS of typical conjugates is associated preponderantly with either of these globulins, possibly as a result of differences in affinity for fluorescein. The study demonstrated also that the technique of gel filtration, which has been used previously for fractionation of antisera not destined for conjugation(5), was a practical method for separation of fluorescein-labeled conjugates into 7S and 19S globulin fractions.

Materials and methods. For preparation of the fluorescein-labeled globulin, rabbit antiserum for *Escherichia coli* 0119:B14 was fractionated by precipitation at 4°C with $(NH_4)_2SO_4$ at a final concentration of 1.95 M. After the globulin was dissolved and reprecipitated twice with this concentration of $(NH_4)_2SO_4$, it was freed of the salt by dialysis against 0.15 M NaCl and was conjugated with crystalline, chromatographically pure fluorescein isothiocyanate† by a widely employed technique(6). Unreacted fluorescent material was removed from the conjugate by dialysis against 0.15 M NaCl buffered at pH 7.5 with 0.01 M phosphate (PBS).

Gel filtration was carried out with glass columns of 2.2 cm internal diameter which were packed to a height of 27 cm with Sephadex G-200‡ in equilibrium with PBS. Two ml of conjugate were applied to each column. The conjugate was eluted with PBS at a hydrostatic pressure of 12.5 cm of buffer. Emergence of conjugate from the columns was monitored with an ultraviolet lamp. The first 4 ml of conjugate passing through each column were pooled, as were the next 2, 12, 10, and 80 ml (Pools I through V, respectively). Pools were concentrated by dialysis against polyethylene glycol 20,000§(7), and then were equilibrated with PBS by dialysis.

Rather than the subjective estimation of fluorescence intensity usually employed, NSS was determined quantitatively by the following procedure(8). Methanol-fixed smears of uninfected KB tissue culture cells were stained with the pooled fractions from the Sephadex columns. NSS was measured with a Model 520-M photometer‖, the search unit of which was modified to fit over the vertical eyepiece of a Leitz Ortholux microscope. The fluorescence assembly was fiitted with an Osram HBO-200 mercury arc lamp, a Schott BG-12 (3 mm) primary filter, and a Schott OG-1 (2 mm) barrier filter. Two smears of KB cells were stained with each fraction. Measurements of the resulting fluorescence of each smear were taken from 10 micro-

* Use of trade name is for identification only and does not constitute endorsement by the Public Health Service or by the U. S. Department of H. E. W.

† Baltimore Biological Laboratory, Baltimore, Md.

‡ Pharmacia Fine Chemicals, Inc., New Market, N. J.

§ Fisher Scientific Co., Fairlawn, N. J.

‖ Photovolt Corp., New York, N. Y.

TABLE I. Staining Activities by Defined Fractions of Varying Protein Concentration from 2 ml of Fluorescent Antibody Conjugate.

Pool	Elution vol (ml)	Total protein (mg)	Svedberg units (S)	F:P ratio*	ml to which concentrated for fluorescence microscopy	Specific fluorescent antibody titer†	NSS‡	Ratio of specific fluorescent antibody titer† to NSS‡
I	4	1.7	19S	22	1.3	8	4	2:1
II	2	1.1	19S, trace of 7S	18	.7	16	6	3:1
III	12	6.9	7S	12	2.0	128	6	21:1
IV	10	4.6	7S	14	2.0	64	7	9:1
V	80	1.7	5S	14	2.0	16	6	3:1
Original conjugate	—	17.8 (per 2 ml)	7S and 19S	14	—	1024	20	51:1

* F:P ratio expressed as μg fluorescein isothiocyanate per mg protein.
† Reciprocal of highest dilution giving bright specific fluorescence with *E. coli*.
‡ Nonspecific staining activity in terms of relative photometric units per KB tissue culture cell.

scopic fields, each containing from 60 to 120 cells. Division of the arithmetic mean of the photometric measurements from the 10 fields by the mean cell count of those fields permitted expression of the NSS in terms of relative photometric units per cell. Previously, it was shown that the precision of these measurements was in excess of that needed to give statistically significant data at 95% confidence limits.

Fluorescein to protein (F:P) ratios and fluorescent antibody staining titers of the reagent pools were determined as detailed previously(9). The protein composition of the pools was studied by cellulose acetate strip electrophoresis (CASE) with the Shandon electrophoresis apparatus,¶ employing a borate buffer and 0.4 ma per cm strip width. Analytical ultracentrifugation of the fractions in PBS was done at 59,780 rpm with a Spinco model E ultracentrifuge** and standard 12 mm cells. The ultracentrifugation was carried out at 20°C, and all sedimentation coefficients are reported as uncorrected Svedberg units (S).

Results. Separation of 7S globulin from 19S globulin of the conjugate was achieved by gel filtration. Ultracentrifugal analysis of the eluted pools after concentration showed them to be composed of globulins with the following S values: Pool I, 19S; Pool II, 19S plus a slight amount of 7S; Pools III and IV, 7S; and Pool V, 5S (Table I). Only 7S and 19S globulins were identified by ultracentrifugation of the unfractionated conjugate, the concentration of 5S protein probably being too small for formation of a distinct peak.

Total protein recovery from the Sephadex columns following concentrations of the fractions was 90% of that applied.

Upon examination by CASE, each concentrated pool produced only 1 band. The protein of Pool V migrated as fluorescein-labeled beta globulin, while the migration rate of the proteins in the other pools was that of labeled gamma globulin. The migration rate of the 7S globulin was indistinguishable from that of the 19S globulin. The unfractionated conjugate produced 2 bands, corresponding to labeled beta and gamma globulins. The migration rate of labeled globulin was greater in every instance than that of the corresponding unlabeled globulin.

The conjugate pools were tested for NSS of KB cells after concentration of each pool to a volume equal to that of the conjugate before fractionation. These concentrations were used in order that the amount of staining by each pool represented the contribution of the protein contained in that pool to the staining by the original conjugate. Since the protein of Pool I and almost all protein of Pool II were 19S globulin, these pools were concentrated so that the sum of their volumes was 2 ml. Results showed that each labeled globulin contributed a significant amount of NSS to that produced by the

¶ Consolidated Laboratories, Inc., Chicago Heights, Ill.

** Beckman Instruments, Inc., Belmont, Calif.

TABLE II. Staining Activities by Pure 7S and 19S Globulin Fractions of Equal Protein Concentration* from Fluorescent Antibody Conjugate.

Pool†	Svedberg units (S)	Specific fluorescent antibody titer‡	NSS§	Ratio of fluorescent antibody titer to NSS
I	19S	32	5	6:1
III & IV combined	7S	128	5	26:1
Original conjugate	7S and 19S	64	7	9:1

* All fractions adjusted to 2.1 mg protein per ml.
† Pools II and V, which were not pure 7S or 19S globulin, not included.
‡ Reciprocal of highest dilution giving bright specific fluorescence with *E. coli*.
§ Nonspecific staining activity in terms of relative photometric units per KB tissue culture cell.

whole conjugate (Table I). Furthermore, upon adjustment of the pools to the same protein concentration, the amount of NSS given by each was practically identical.

Specific staining, however, was associated predominantly with 7S gamma globulin (Table I). The ratio of specific staining titer to NSS was 2:1 for the 19S globulin of Pool I and 21:1 for the 7S globulin of Pool III. The original conjugate showed the most favorable ratio (51:1) by virtue of its higher specific staining titer. No pool had a specific titer as high as that of the original conjugate because the 7S globulin initially present was distributed among more than 1 pool. Pools III and IV, which contained practically all of the 7S globulin from the original conjugate, were combined and compared with the unfractionated conjugate adjusted to the same protein concentration. The ratio of specific staining titer to NSS with these combined pools of pure 7S globulin was 26:1 (Table II). At the same protein concentration, the ratio was only 9:1 for the unfractionated conjugate, which contained its original 7S, 19S, and 5S globulin components.

Comparison of the data of Table I with those of Table II shows that the ratio of specific fluorescent antibody titer to NSS by a fraction varied markedly with the protein concentration at which the determinations were made. Since it has been observed repeatedly in this laboratory that these two staining activities do not change to the same extent upon dilution of a conjugate, the observation was not unexpected.

Discussion. The presence in immunofluorescent reagents, such as those prepared from whole serum, of labeled protein that contributes to NSS but not to specific staining is obviously undesirable. A widely used method to decrease the concentration of this non-antibody protein is fractionation of the antiserum with $(NH_4)_2SO_4$ prior to conjugation. The present study indicated that conjugates prepared from antisera fractionated by $(NH_4)_2SO_4$ under carefully standardized conditions were composed largely of labeled gamma globulin. Nevertheless, undesirable levels of NSS occur frequently with these conjugates. Reduction of the level of NSS by dilution of conjugates is permissible only when the original specific staining titer is high. Sorption with tissue powders or tissue homogenates sacrifices considerable specific antibody protein and is of limited effectiveness in decreasing NSS(10). A more practical solution may be the selective removal of non-antibody protein that remains despite fractionation of the antiserum with $(NH_4)_2SO_4$ prior to conjugation. The present study showed that labeled 19S globulin was removed readily from conjugates for *E. coli* by gel filtration. This 19S globulin was responsible for a considerable amount of NSS and contributed little to specific staining. The specific staining activity was high in fractions in which only 7S globulin was detected.

Column chromatography with diethylaminoethyl (DEAE) cellulose also may be used to obtain 7S globulin fractions from fluorescein conjugates(11). Routine use of this technique is discouraged by disadvantages such as the lengthy process required to remove impurities from the resin as obtained commercially, the exhaustive washing of the cleansed resin necessary to establish equilibrium with the elution buffer, and the large

dilution factor inherent to ion-exchange chromatography. Molecules heavily labeled with fluorescein, which have been incriminated as a cause of NSS, may be removed by DEAE chromatography, but not by gel filtration. However, the importance of this function is diminished by recent technical advances that allow the production of conjugates possessing practically any F:P ratio desired(4).

Elimination of 19S globulin from conjugates is advantageous only when this globulin makes no important contribution to specific staining. Numerous studies have been made to determine the types of globulin with which antibody is associated. The distribution varies with the particular antigen employed, the species of animal immunized, and the intensity of immunization(12). Fractionation of conjugates by gel filtration as described in the present study should prove useful in establishing the effect of these variables upon the type of globulin in which antibody appears.

Summary. Gel filtration proved to be a simple, effective method for separation of fluorescein-labeled antiglobulin for *E. coli* into 7S and 19S fractions. Both fractions were found to contribute considerable nonspecific staining, while specific staining was associated predominantly with the 7S globulin. The ratio of specific staining titer to nonspecific staining activity was 3-fold greater with a pool of fractions in which only 7S globulin was detected than with the unfractionated conjugate when the 2 preparations were tested at the same protein concentration.

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Metabolism of Labelled Steroid Precursors by Normal Bovine Adrenal Medulla *in vitro*.* (30291)

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Experimental evidence has recently been adduced supporting the view that chromaffin tissue possesses to a significant degree the enzymatic actions of the adrenal cortex at several stages of the currently accepted scheme of steroid biosynthesis(1,2). This suggestion was put forward as a result of our studies with chromaffin tumors incubated with various radioactive precursors. The 3 major steroid hydroxylases (21, 11β and 17α), the

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